## Title

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# Requirement of the NPXY Motif in the Integrin β3 Subunit Cytoplasmic Tail for Melanoma Cell Migration In Vitro and In Vivo

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***Abstract.*** The NPXY sequence is highly conserved among integrin β subunit cytoplasmic tails, suggesting that it plays a fundamental role in regulating integrin-mediated function. Evidence is provided that the NPXY structural motif within the β3 subunit, comprising residues 744–747, is essential for cell morphological and migratory responses mediated by integrin αvβ3 in vitro and in vivo. Transfection of CS-1 melanoma cells with a cDNA encoding the wild-type integrin β3 subunit, results in de novo αvβ3 expression and cell attachment, spreading, and migration on vitronectin. CS-1 cells expressing αvβ3 with mutations that disrupt the NPXY sequence interact with soluble vitronectin or an RGD peptide, yet fail to attach, spread, or migrate on immobilized ligand. The biological consequences of these observations are underscored by the finding that CS-1 cells expressing wild-type αvβ3 acquire the capacity to form spontaneous pulmonary metastases in the chick embryo when grown on the chorioallantoic membrane. However, migration-deficient CS-1 cells expressing αvβ3 with mutations in the NPXY sequence lose this ability to metastasize. These findings demonstrate that the NPXY motif within the integrin β3 cytoplasmic tail is essential for αvβ3-dependent post-ligand binding events involved in cell migration and the metastatic phenotype of melanoma cells.

Cell adhesion and migration play a central role in diverse biological and pathological processes including embryogenesis, angiogenesis, wound healing, and metastasis. Although the mechanisms that regulate cell adhesion and motility are poorly understood, it is clear that in many instances, these events are initiated by the specific recognition of extracellular matrix proteins by a family of cell surface adhesion receptors known as integrins (Ruoslahti, 1991; Hynes, 1992). Upon ligand recognition, integrins typically cluster at the site of cell–substrate interface; this leads to polymerization of the actin cytoskeleton (Burridge et al., 1988). In this context, integrins form a physical linkage between the extracellular matrix and the actin cytoskeleton. Ultimately, these integrin-initiated adhesive interactions can lead to gross changes in cellular morphology orchestrated by the actin cytoskeleton that facilitate cell migration.

Structurally, integrins are heterodimers composed of noncovalently associated α and β subunits (Hynes, 1992). Each subunit contains a single transmembrane domain that separates a large ectodomain from its cytoplasmic tail, which in most cases is shorter than 50 amino acids. Although several studies have identified functionally important regions within the integrin ectodomain (Smith and Cheresh, 1988, 1990; D'Souza et al., 1988, 1990, 1994; Loftus et al., 1990; Takada et al., 1992; Lee et al., 1995) and cytoplasmic domain (Hibbs et al., 1991; O'Toole et al., 1991, 1995; Bauer et al., 1993; Filardo and Cheresh, 1994) that can regulate ligand-binding function, relatively little is known regarding structural motifs within the integrin that regulate morphological changes in cell shape which accompany cellular adhesion and migration.

Several observations indicate that, upon ligand binding, the integrin cytoplasmic tail mediates a physical interaction with cytoskeletal proteins (Otey et al., 1990, 1992; Miyamoto et al., 1995). These interactions are presumed to promote the organization of the actin cytoskeleton leading to changes in cellular structure and morphology associated with integrin receptor clustering at focal contacts. Moreover, several studies have reported that sequences within the β subunit cytoplasmic tails may play a role in the recruitment of integrins to cell–substrate attachment sites (Hayashi et al., 1990; La Flamme et al., 1992; Reszka et al., 1992; Ylänne et al., 1993; Cone et al., 1994). These sequences represent regions of structural homology between the individual integrin β subunit cytoplasmic tails including a membrane-proximal sequence

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that forms an amphipathic helix, and two distal well-conserved sequences, NPXY and NXXY, that comprise tight β turns. While these regions have been linked to the ability of integrins to localize to focal contacts, little is known about these domains in terms of postligand binding events.

Integrin αvβ3 is highly expressed on various motile cells in vivo, including neural crest cells (Delannet et al., 1994), vascular endothelial cells (Brooks et al., 1994*a*,*b*), and malignant melanoma cells (Albelda et al., 1991). Therefore, to define structural regions within the β3 cytoplasmic tail that are required for αvβ3 post-ligand binding function, we utilized a hamster cell line, CS-1, that fails to express endogenous β3 or β5 subunit proteins, and as a consequence does not display any vitronectin receptors on its surface (Thomas et al., 1993). Upon transfection of a cDNA-encoding wild-type β3 subunit protein, these cells express αvβ3 enabling them to attach, spread, and migrate in response to vitronectin. Moreover, these cells gain the ability to form pulmonary metastases in the chick embryo after being grown on the chorioallantoic membrane (CAM)[1]. In contrast, mutations that disrupt the NPXY sequence (amino acids 744–747) present in the β3 cytoplasmic tail maintain their capacity to bind soluble ligand yet are unable to promote αvβ3-dependent cell morphological changes that are prerequisite for cell motility both in vitro and during tumor cell metastasis in vivo.

## Materials and Methods

### Cell Culture

The CS-1 hamster melanoma cell line was originally described by Knudsen et al., 1982. These cells do not express αvβ3 or αvβ5 vitronectin receptors, since they fail to express β3 or β5, and as a consequence are nonadherent when cultured in the presence of serum (Thomas et al., 1993). CS-1 cells were propagated in RPMI media supplemented with 5% FBS, 2 mM L-glutamine, and 50 μg/ml gentamicin.

### cDNA Constructs

Mutant and wild-type integrin β3 subunit proteins were generated from a molecular clone, pβ3GEM-1, containing a 3.8-kb EcoRI cDNA fragment encoding a full-length human β3 polypeptide (Fitzgerald et al., 1987). To express wild-type β3 polypeptide, the EcoRI ends of the 3.8-kb pβ3GEM-1 cDNA fragment were filled in with Klenow enzyme, and blunt-end ligated into the EcoRV site within the polylinker of the pcDNA-1neo vector (Invitrogen, La Jolla, CA). To facilitate the construction of cDNAs encoding mutant β3 cytoplasmic tails, an expression cassette encoding the amino-terminal two thirds of the β3 subunit polypeptide was created within the pcDNA-1neo expression vector. An oligonucleotide containing a HindIII restriction site, d(CC**AAGCTT**GG), was inserted into the 5′ EcoRI site of the pβ3GEM-1 cDNA and the 1,500-bp HindIII-BamHI β3 fragment was directionally cloned into pcDNA-1neo to generate pH3Bm1.5β3neo.

Truncated β3 subunit proteins are named based upon the position of their COOH-terminal amino acid residue with respect to the mature β3 polypeptide and are designated by the symbol Δ. To construct cDNAs encoding β3.751 Δ- and β3.741 Δ-truncated polypeptides, cDNA fragments were amplified from linearized pβ3GEM-1 plasmid DNA by PCR using an upstream sense primer corresponding to nucleotides (nts) 2139-2165 and downstream anti-sense primers 5′ CTAG**TCTAGA**CTAGGTGGCCTCTTTATACAGTGGGTTG 3′, β3.751(Δ) and 5′ CTAG**TCTAGA**CTAGGTGTCCCATTTTGCTCTGGCGCGTTC 3′, β3.741(Δ) containing nts 2295–2320 and 2327–2351, respectively. In addition to the β3-derived nucleotides, the downstream primers were designed to introduce an amber stop codon (underlined) and an XbaI restriction endonuclease recognition site (bold) in order to facilitate the exchange of these PCR fragments into the pcDNA-1neo vector. EcoRI-XbaI–restricted PCR fragments encoding the truncated cytoplasmic tails were then purified by electrophoresis through 8% nondenaturing acrylamide gels, and ligated in a ternary reaction with a 774-bp BamHI-EcoRI fragment encoding the remainder of the ectodomain and the transmembrane anchor nt 1500-2274 and BamHI-XbaI–digested pH3Bm1.5β3neo.

Alanine or phenylalanine substitution mutants are designated by the position of the amino acid residue at which the substitution has been introduced into the wild-type β3 polypeptide. The first letter following the number indicates the original residue and the second letter following the / denotes the substituted one. To create a mutagenesis cassette, the 1,136-kb BamHI-SacI fragment encoding the carboxyl-terminal one third of the β3 subunit protein, nt 1500–2636, was subcloned into pTZ18U (U.S. Biochem. Corp., Cleveland, OH). The 3′ SacI site at nt 2636 in the untranslated region was converted to an XbaI site by the insertion of a synthetic oligonucleotide d(CTAG**TCTAGA**CTAG) (New England Biolabs, Beverly, MA) to create 18Uβ31.1X. Amino acid substitutions were produced by oligonucleotide mutagenesis of uracylated phagemid DNA prepared from 18Uβ31.1X as described by Filardo and Cheresh (1994). Mutant cDNA clones were identified by DNA sequence analysis using the dideoxynucleotide chain termination method (Sanger et al., 1977). Mutagenized 1136-bp BamHI-XbaI fragments were isolated and ligated to Bam HI-XbaI-digested pH3Bm1.5β3neo to generate cDNAs encoding full-length β3 polypeptides containing individual point mutations within the cytoplasmic tail.

### Monoclonal Antibodies

mAb LM609 (αvβ3) has been previously described (Cheresh and Spiro, 1987). mAb AP3, specific for the human β3 subunit, was a generous gift of Dr. Peter Newman (Blood Center of Southwestern Wisconsin, Milwaukee, WI). Fluoresceinated ligand induced binding site (LIBS)-1 detects a conformer-specific epitope on β3 integrins (Frelinger et al., 1990) and was kindly provided by Dr. Mark Ginsberg (Scripps Research Institute, La Jolla, CA). mAb 7E2 specific for hamster β1 integrins was graciously provided by Dr. Rudolph Juliano (University of North Carolina, Chapel Hill, NC).

### Transfection and Selection of Stable β3-expressing Cell Lines

β3-deficient CS-1 cells were transfected with pcDNA-1neo constructs encoding cDNAs encoding full-length or mutated versions of the β3 subunit protein using lipofectin (GIBCO BRL, Gaithersburg, MD). 3 d after transfection, 500 μg/ml of geneticin (Sigma Chemical Co., St. Louis, MO) was added to the growth medium. Transfected cells expressing mutant or wild-type β3 integrins were enriched from the geneticin resistant population by fluorescence activated cell sorting using β3-specific mAb, AP3. Transfectants were sorted based on their mean intensity fluorescence, where the highest staining (0.5%) cells were gated under sterile conditions, expanded in culture, and then resorted. This procedure was repeated until stable cell lines were obtained that displayed equivalent amounts of wild-type or mutated β3 integrins on their surface. The integrity of each mutation within the β3 cytoplasmic tail was verified by nucleotide sequence determination of PCR products generated from polyadenylated RNA synthesized in each transfected cell line. Throughout the course of these studies, all cell lines were maintained free of mycoplasma.

### Ligands and Cellular Adhesion Assays

Vitronectin was purified from human serum as previously described (Yatohgo et al., 1988). Mouse laminin was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Cellular adhesion assays were performed as previously described (Klemke et al., 1994).

### Spreading on Integrin Specific Antibodies

Glass coverslips (12 mm, No.1 thickness) were coated with 20 μg/ml goat anti-mouse immunoglobulin (Southern Biotechnology Associates, Birmingham, AL) for 1 h at room temperature. Coverslips were then washed twice in PBS, and blocked with 1% heat denatured BSA for 1 h at 37°C. The wells were then washed with PBS and incubated in 10 μg/ml of AP3 mAb for 1 h at room temperature, and washed twice in adhesion buffer. β3 transfected cells were seeded at a density of 100,000 cells/ml and incu-

1. *Abbreviations used in this paper*: CAM, chorioallantoic membrane; LIBS, ligand induced binding site; nt, nucleotide; WT, wild-type.

bated at 37°C in a humidified atmosphere for 2 h. Samples were viewed using a microscope equipped with phase contrast (Nikon Inc., Instr. Group, Melville, NY).

### *RGD-induced LIBS-1 Expression*

CS-1 transfectants expressing wild-type or mutant β3 integrins were analyzed for RGD-induced LIBS-1 expression as previously reported (Filardo and Cheresh, 1994). GRGDSPK and SPGDRGK peptides were provided by E. Merck (Darmstadt, Germany).

### *Cellular Migration Assays*

Cell migration assays were performed using modified Boyden chambers containing polycarbonate membranes (tissue culture treated, 6.5-μm diameter, 10-μm thickness, 8-μm pore, Transwell; Costar Corp., Cambridge, MA). Migration toward immobilized adhesive ligand (haptotaxis) was measured as described by Klemke et al. (1994). Migration toward soluble ligand (chemotaxis) was assessed in a similar manner. Except in this case, the undersurface of the membrane and all surfaces of the lower reservoir were preblocked with BSA. Excess BSA was removed and the lower reservoir was filled with 0.5 ml of FBM containing vitronectin or laminin (10 μg/ml). Cells that migrated into the lower chamber were enumerated by counting the number of cells on the floor of the lower reservoir per 200× field.

### *Chick Embryo Metastasis Assay*

The chick embryo metastasis assay was performed as previously described with some minor modifications (Brooks et al., 1993). Five million CS-1 melanoma cells expressing wild-type or mutant β3 integrins were deposited on the CAM of 10-d-old chick embryos and incubated at 39°C in a humidified atmosphere. After 1 wk, tumors formed at the primary site were excised, trimmed free of surrounding CAM tissue, and wet weights were determined. Tumor invasion was assessed by determining the percentage of melanoma cells present in a single cell suspension of whole lung tissue by flow cytometry using mAb 7E2, specific for hamster β1 integrins.

## *Results*

### *Residues 742–751 within the β3 Cytoplasmic Tail Are Required for αvβ3-dependent Cellular Adhesion and Spreading*

Comparison of the cytoplasmic tail sequences of the individual integrin β subunits reveals a striking degree of structural homology. A structural motif common to six of the eight β subunit cytoplasmic tails is the presence of two well-conserved predicted tight β turns (see Fig. 1). One of these β turns, defined by the NPXY sequence, has been shown to have an influence on the recruitment of transfected integrins into preformed focal contacts (Reszka et al., 1992; Cone et al., 1994). Therefore, to address the influence of the NPXY sequence with respect to its function in regulating integrin-mediated adhesion, we examined the effect of mutations that eliminate the NPXY structural motif from the cytoplasmic domain of integrin αvβ3.

The adhesive properties of cells expressing αvβ3 containing full-length or truncated β3 subunit proteins were characterized by assessing the relative capacities of the β3.741 (Δ), β3.751 (Δ), and β3.762 (WT) cell lines to adhere to polystyrene wells coated with varying concentrations of vitronectin or the control protein, laminin. As shown in Fig. 2 *A*, nontransfected CS-1 cells, lacking αvβ3, were incapable of attaching to vitronectin, yet attached well to the laminin-coated surface, consistent with their expression of β1 integrins (Thomas et al., 1993). CS-1 cells expressing αvβ3 composed of β3.762 (wild-type) or β3.751

| | | | | | |
|---|---|---|---|---|---|
| β1 | IIHDRREFAKFEKEKMNAKWDTGE | **NPIY** | KSAVTTVV | ***NPKY*** | EGK |
| β2 | HLSDLREYRRFEKEKLKSQWNND- | **NPLF** | KSATTTVM | ***NPKF*** | AES |
| β3 | TIHDRKEFAKFEEERARAKWDTAN | **NPLY** | KEATSTFT | ***NITY*** | RGT |
| β5 | TIHDRREFAKFQSERSRARYEMAS | **NPLY** | RKPISTHTVDFTFNKF | ***NKSY*** | NGTVD |
| β6 | SFHDRKEVAKFEAERSKAKWQTGT | **NPLY** | RGSTSTFK | ***NVTY*** | KHREKQKVDLSTDC |
| β7 | EIYDRREYSRFEKEQQQLNWKQDS | **NPLY** | KSAITTTI | ***NPRF*** | QEADSPTL |

*Figure 1.* Alignment of the deduced amino acid sequences of several integrin β subunit cytoplasmic tails. The deduced amino acid sequences of the β subunit cytoplasmic tails listed above are obtained from the following published data: β1 (Argraves et al., 1987), β2 (Kishimoto et al., 1987), β3 (Fitzgerald et al., 1987), β5 (McLean et al., 1990), β6 (Sheppard et al., 1990), and β7 (Erle et al., 1991). For each β subunit, the first amino acid listed is the residue that follows the transmembrane anchor. Residues comprising the tight β turns encoded by the **NPXY** (*underscored*) and **NXXY** (*italicized*) sequences are denoted.

(Δ) polypeptides adhered and spread well on vitronectin, indicating that elimination of 11 COOH-terminal amino acids, including the NXXY predicted β turn (residues 756–759) had little, if any, effect on αvβ3-mediated cell attachment. Adhesion of either of these β3-transfected cells to vitronectin was completely inhibited by mAb LM609 demonstrating that αvβ3 is responsible for this event (Filardo, E. J., and D. A. Cheresh, unpublished data). In contrast, CS-1 cells displaying αvβ3 containing the β3.741 (Δ)-truncated subunit protein failed to attach to the vitronectin substrate, while they readily attached to laminin. These results suggest that the sequence between $Thr^{741}$ and $Ala^{750}$ is required for αvβ3-mediated cell attachment to immobilized vitronectin.

Cellular adhesion to planar surfaces is greatly strengthened by cell spreading (Lotz et al., 1989). Therefore, to establish whether the failure of CS-1 cells expressing β3.741 (Δ) to attach to a vitronectin substrate may be due in part to an inability of these cells to organize their actin cytoskeleton and promote changes in cell shape, cells expressing wild-type or mutant αvβ3 were allowed to attach to coverslips coated with mAb AP3, specific for the β3 subunit protein. As shown in Fig. 2 *B*, all cell lines expressing αvβ3 attached to the anti-β3 antibody, but only αvβ3.762 (WT) and αvβ3.751 (Δ) cells were able to spread on this ligand. In contrast, αvβ3.741 (Δ) cells attached but remained rounded, indicating their inability to engage the cytoskeleton and promote a shape change. These data suggest that amino acids 742–751 of the β3 subunit play a critical role in regulating αvβ3-dependent cell spreading, and may explain why cells expressing this form of αvβ3 fail to maintain an adherent phenotype on vitronectin.

### *Residues $Asn^{744}$ or $Tyr^{747}$ within the NPXY Sequence in the β3 Cytoplasmic Tail Are Essential for αvβ3-dependent Cellular Shape Changes*

Theoretical calculations that predict protein secondary structure (Chou and Fasman, 1978; Rose, 1978) indicate that the NPXY sequence within the integrin β subunit cytoplasmic tails forms a tight turn. To determine whether this structural motif, defined by residues $Asn^{744}$-$Tyr^{747}$ of the β3 cytoplasmic tail, is necessary for αvβ3-mediated

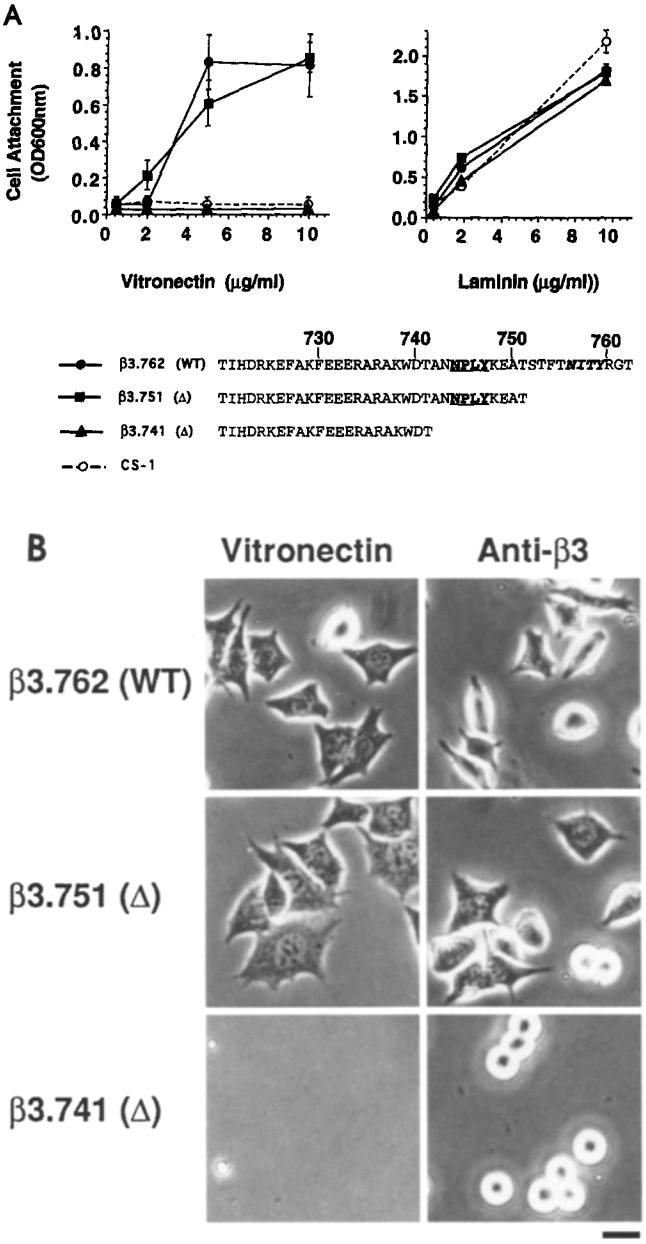


*Figure 2.* The effect of β3 truncation on CS-1 cell attachment to vitronectin. (*A*) CS-1 cells expressing αvβ3 consisting of full-length (β3.762) or truncated (β3.751Δ or β3.741Δ) β3 subunit proteins were allowed to attach for 2 h to wells coated with varying concentrations of vitronectin or laminin. After adhesion, unattached cells were removed by washing, and the attached cells were stained with crystal violet. Cell attachment was quantified by spectrophotometric analysis at OD 600 nm of the crystal violet dye eluted from the adherent cells. Each data point represents the mean ± the standard deviation of quadruplicate samples. Nonspecific cell adhesion as measured on BSA-coated wells has been subtracted. (*B*) Phase-contrast micrographs of CS-1 cells expressing wild-type, β3.762 (WT) or truncated, β3.751 (Δ) and β3.741 (Δ) polypeptides plated on 10 μg/ml of either vitronectin or anti-β3 mAb, AP3. 300×. Bar, 10 μm.

cellular adhesion to vitronectin, individual amino acid residues within this region were substituted by alanine-scanning mutagenesis. The adhesive properties of cells expressing αvβ3 containing single alanine substitution between amino acid residues 741–750 in the β3 subunit cytoplasmic tail were examined on varying concentrations of immobilized vitronectin. As shown in Fig. 3 *A*, CS-1 cells expressing αvβ3 with alanine substitutions in residues immediately upstream, $Asn^{743}$ or downstream, $Lys^{748}$ of the $NPXY^{744-747}$ sequence, exhibited vitronectin adhesion activity comparable to cells expressing wild-type αvβ3. Similarly, conversion of $Pro^{745}$ to an alanine had no effect on vitronectin binding. In contrast, replacement of $Asn^{744}$ with an alanine resulted in a total loss of vitronectin adhesion behaving identically to truncation mutant β3.741 (Δ) (Fig. 2). This mutant cell line did not adhere to surfaces coated with as much as 20 μg/ml of vitronectin (data not shown). In addition, an alanine substitution in $Tyr^{747}$ exhibited a substantial but not complete loss in vitronectin adhesive activity (Fig. 3 *A*). The finding that two independent point-mutations in noncontiguous residues within the NPXY sequence impacted vitronectin adhesive function suggests that the structural integrity of this region is required for β3 integrin function.

Reduced vitronectin adhesivity displayed by αvβ3.747 Y/A cells may be due either to alterations in the structure of this region or a loss of phosphorylation at this site. To address this issue, the tyrosine at position 747 was substituted with phenylalanine, an amino acid which is structurally homologous to tyrosine, but lacks the hydroxyl group which is the acceptor site for phosphorylation. As shown in Fig. 3 *A*, cells expressing αvβ3.747 Y/F containing a phenylalanine in place of $Tyr^{747}$ exhibited wild-type vitronectin adhesion, supporting the idea that it is the structure of this region rather than its phosphorylation status that is essential for adhesion to this substrate.

These same point mutants were examined for their ability to spread on immobilized anti-β3 antibody. Cells expressing αvβ3 containing individual amino acid substitutions that were capable of adhering to vitronectin were able to spread on this substrate or anti-β3 antibody (Fig. 3 *B*). Conversely, point mutants defective in their capacity to adhere to vitronectin (Fig. 3 *A*) were unable to spread on immobilized anti-β3 antibody (Fig. 3 *B*), consistent with their inability to engage the actin cytoskeleton and mediate alterations in cell shape. Collectively, these findings demonstrate that the structure defined by the NPXY sequence within the β3 cytoplasmic tail is required to induce αvβ3-dependent cellular shape change.

### *Effect of Mutations within the β3 Cytoplasmic Tail on Cell Interaction with Soluble Vitronectin or an RGD Peptide*

To determine whether mutations within the β3 NPXY sequence may alter the ligand binding properties of αvβ3, cells expressing these mutant receptors were examined for their ability to bind soluble ligand. Ligand occupancy of β3 integrins can be measured by a conformational change in the β3 subunit as detected by the LIBS-1 monoclonal antibody (Frelinger et al., 1990). Therefore, expression of the LIBS-1 epitope on αvβ3.741 (Δ), αvβ3.744 N/A, or αvβ3.762 (WT) cells was measured in the presence or absence of soluble GRGDSPK heptapeptide, a known ligand for αvβ3 (Spiro and Cheresh, 1987). As shown in Fig. 4 *A*, all three cell lines expressed equivalent LIBS-1 reactivity in the presence of 500 μM GRGDSPK peptide, yet failed

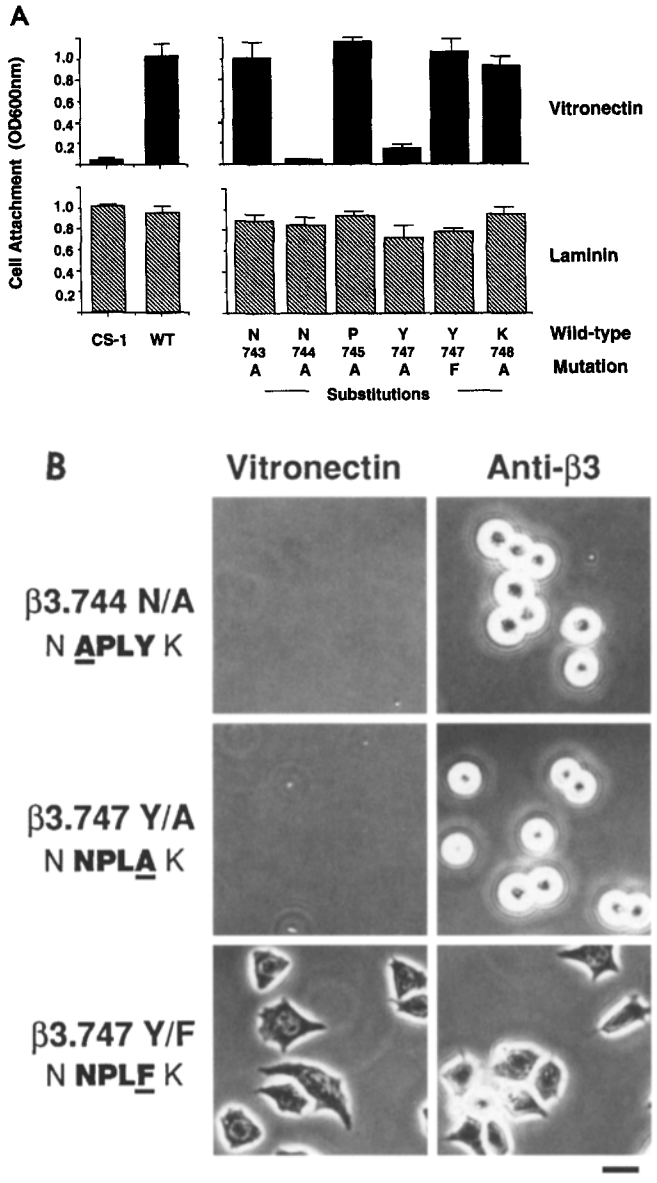


*Figure 3.* Adhesive properties of CS-1 cells expressing αvβ3 with alanine or phenylalanine substitutions within the β3 subunit cytoplasmic tail. (*A*) CS-1 cells expressing αvβ3-containing amino acid substitutions in the residues encompassing the β turn encoded by the NPXY sequence (744–747) in the β3 subunit cytoplasmic tail were allowed to attach for 2 h to wells coated with 10 μg/ml of vitronectin (*black*) or laminin (*gray*). After adhesion, the attached cells were quantified as described in Fig. 2. (*B*) Phase-contrast micrographs of CS-1 cells expressing β3 polypeptides containing individual amino acid substitutions, β3.744 N/A, β3.747 Y/A, or β3.747 Y/F plated on coverslips coated with either vitronectin or anti-β3 mAb, AP3. 300×. Bar, 10 μm.

to show LIBS-1 binding in the presence of the same concentration of the scrambled control peptide SPGDRGK. More importantly, each of these β3-transfected cell lines showed an equivalent RGD dose-dependent LIBS-1 activation with half-maximal concentrations between 1 and 2 μM, suggesting that RGD binds to each of these receptors with comparable affinity (Fig. 4 *B*). We next examined whether intact soluble vitronectin binding to these cells would induce expression of the LIBS-1 epitope. As demonstrated in Fig. 4 *C*, αvβ3.741Δ, αvβ3.744 N/A, or αvβ3.762 (WT) cells bound soluble vitronectin as measured by LIBS-1 expression. These data demonstrate that mutations in the β3 cytoplasmic tail that eliminate or disrupt the NPXY sequence abolish αvβ3 cell attachment to immobilized vitronectin, yet do not perturb the ability of αvβ3 to interact with soluble ligands.

### *The Role of the β3 NPXY Sequence in Melanoma Cell Motility In Vitro and Metastasis In Vivo*

Integrin αvβ3 has been shown to promote tumor cell migration (Leavesley et al., 1992). Therefore, to investigate the role of the β3 NPXY sequence in cell motility, cells expressing wild-type or mutant αvβ3 were tested for their chemotactic or haptotactic response to soluble or immobilized vitronectin, respectively. As shown in Fig. 5, *A* and *B*, CS-1 cells lacking αvβ3 fail to migrate toward soluble or immobilized vitronectin while they readily migrate in response to the control protein laminin. Cells expressing either αvβ3.762 (WT) or αvβ3.751 (Δ) acquire the ability to migrate toward lower chambers coated with vitronectin (Fig. 5 *A*) or containing soluble vitronectin (Fig. 5 *B*). In contrast, CS-1 cells expressing αvβ3.741 (Δ) fail to migrate in response to either immobilized or soluble vitronectin. These findings demonstrate that although the αvβ3.741 (Δ) cells bind soluble vitronectin, they are unable to chemotax toward this protein.

Integrin-mediated cell motility events have been linked to cell invasion in vivo (Juliano, 1987; Cheresh, 1991). Furthermore, expression of αvβ3 on the surface of human melanoma cells has been associated with tumorigenicity and progression toward malignancy (Albelda et al., 1991; Felding-Habermann et al., 1992; Nip et al., 1993). To examine whether the NPXY sequence within the β3 cytoplasmic tail can regulate cellular migration in vivo , CS-1 cells expressing wild-type or mutant αvβ3 were examined for their growth and metastatic properties in the chick embryo model (Brooks et al., 1993). CS-1 melanoma cells were deposited onto the CAM of 10-d-old chick embryos and allowed to grow for 1 wk, after which the primary tumor nodule was surgically excised and weighed. In addition, the lungs were harvested, and a single cell suspension was prepared by collagenase treatment. Pulmonary metastasis was quantified by determining the percentage of tumor cells present in this cell suspension by staining with a monoclonal antibody specific for hamster cells. As shown in Fig. 6, CS-1 cells lacking αvβ3, formed large tumors at the primary site, yet these animals contained few, if any, detectable tumor cells in the lungs. In contrast, αvβ3.762 (WT) cells produced small primary tumors which, on average, were fivefold smaller than CS-1 tumors (Fig. 6 *A*). However, as shown in Fig. 6 *B*, this lack of primary tumor size was associated with an approximate fivefold increase in pulmonary metastasis ($P \leq 0.001$), suggesting that expression of αvβ3 on CS-1 cells promoted migration from the primary tumor site. In contrast, cells expressing αvβ3.744 N/A (Fig. 6) or β3.747 Y/A (data not shown), which exhibited deficient migration in vitro, produced tumors whose growth properties and metastatic behavior were identical to the parental, αvβ3-negative CS-1 cell line. CS-1 cells expressing αvβ3.747 Y/F possessing

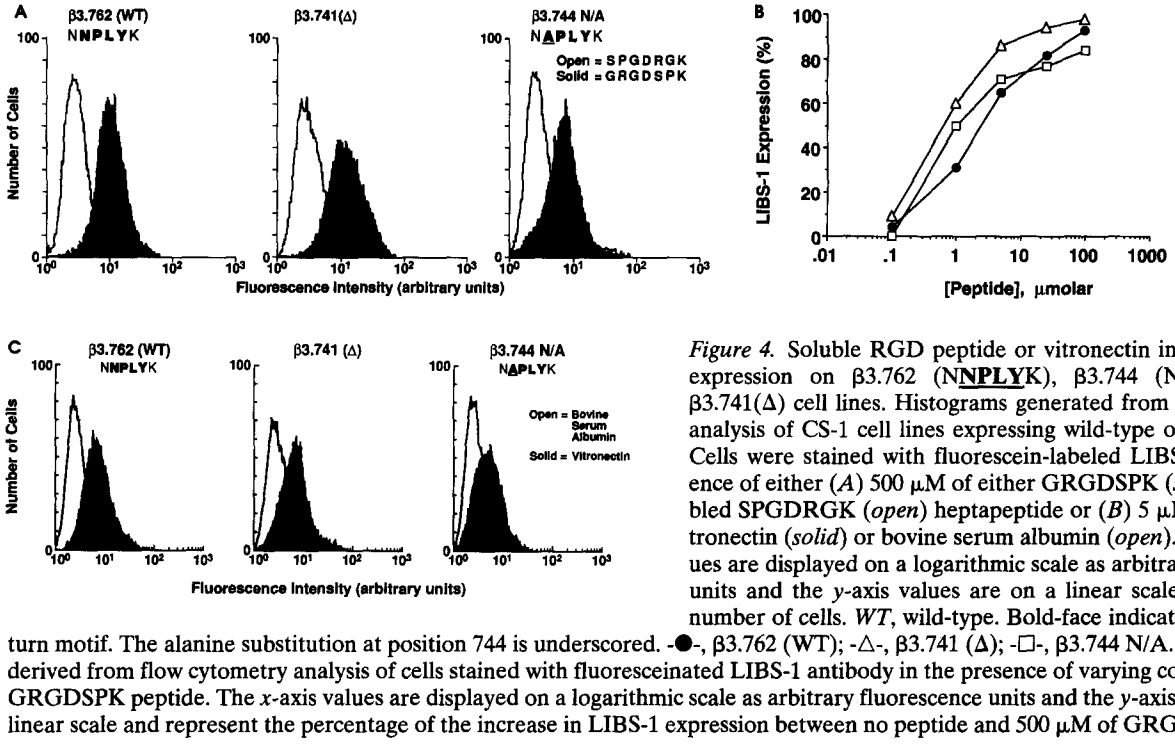


*Figure 4.* Soluble RGD peptide or vitronectin increases LIBS-1 expression on β3.762 (N**NPLY**K), β3.744 (N**APLY**K) and β3.741(Δ) cell lines. Histograms generated from flow cytometry analysis of CS-1 cell lines expressing wild-type or mutant αvβ3. Cells were stained with fluorescein-labeled LIBS-1 in the presence of either (*A*) 500 μM of either GRGDSPK (*solid*) or scrambled SPGDRGK (*open*) heptapeptide or (*B*) 5 μM of soluble vitronectin (*solid*) or bovine serum albumin (*open*). The *x*-axis values are displayed on a logarithmic scale as arbitrary fluorescence units and the *y*-axis values are on a linear scale and represent number of cells. *WT*, wild-type. Bold-face indicates the NPXY β turn motif. The alanine substitution at position 744 is underscored. -●-, β3.762 (WT); -△-, β3.741 (Δ); -□-, β3.744 N/A. (*C*) Line graph derived from flow cytometry analysis of cells stained with fluoresceinated LIBS-1 antibody in the presence of varying concentrations of GRGDSPK peptide. The *x*-axis values are displayed on a logarithmic scale as arbitrary fluorescence units and the *y*-axis values are on a linear scale and represent the percentage of the increase in LIBS-1 expression between no peptide and 500 μM of GRGDSPK.

wild-type migration properties in vitro, completely restored the metastatic phenotype of these cells. The observed inverse correlation between the size of the primary tumor and its ability to metastasize suggests that CS-1 cells lacking the metastatic phenotype are unable to migrate from the primary lesion. In support of this contention, αvβ3 with mutations that disrupt CS-1 melanoma cell motility in vitro also prevent pulmonary metastasis in vivo. Collectively, these data provide evidence that the NPXY sequence within the β3 cytoplasmic tail is a critical structure in regulating cellular migration in vitro and in vivo.

## Discussion

Integrin-mediated adhesion events are associated with a wide range of biological activity both in vitro and in vivo. In fact, integrin gene "knock-outs" lead to embryonic lethal mutations (Yang et al., 1995) and altered cell biologi-

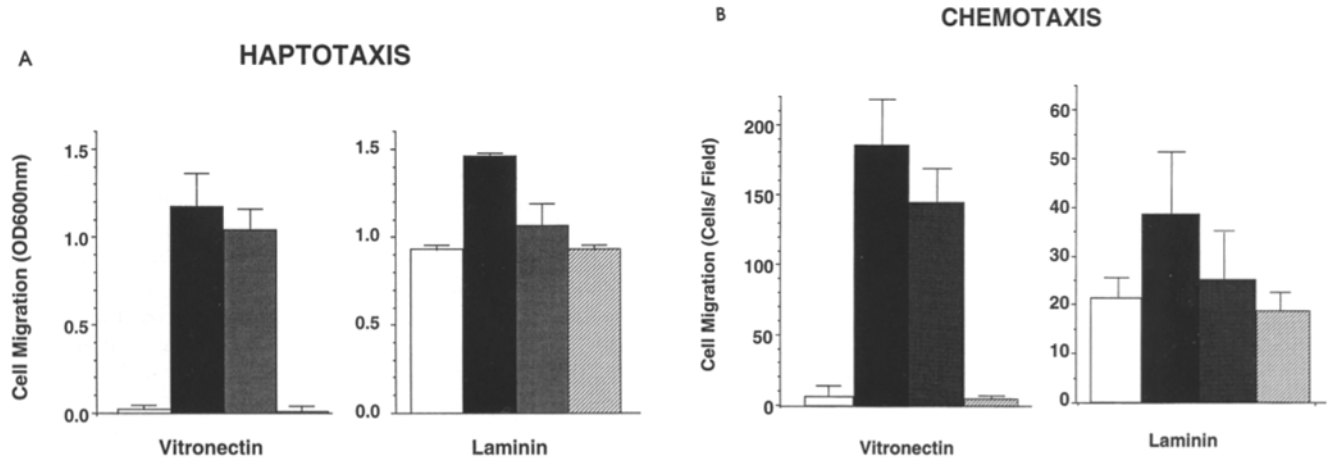


*Figure 5.* Cell migration of CS-1 cells expressing αvβ3 consisting of full-length or truncated β3 polypeptides. Cellular motility toward vitronectin or laminin was determined using a modified Boyden chamber separated by a 6.5 μm porous membrane. CS-1 (*white*), β3.762 (WT) (*black*), β3.751 (Δ) (*gray*), or β3.741 (Δ) (*hatched*) cells were seeded into the upper chambers and allowed to migrate for 24 h toward lower chambers containing 10 μg/ml of immobilized adhesive ligand (*haptotaxis*) (*A*) or soluble ligand (*chemotaxis*) (*B*). Cells which had migrated to the lower reservoir were enumerated by dye uptake (*A*) or by microscopic evaluation (*B*) as described in the experimental procedures. Each bar represents the mean ± the standard deviation of quadruplicate samples.

cal responses (Stephens et al., 1993) while integrin antagonists block platelet aggregation (Savage et al., 1990), tumor cell migration (Humphries et al., 1986), angiogenesis (Brooks et al., 1994), and viral infection (Wickham et al., 1993). The biological role of integrins depends on their ability to bind extracellular matrix proteins resulting in the generation of intracellular signals. This leads to cell spreading and migration, events which have been linked to the organization of the actin cytoskeleton (Burridge et al., 1988). Therefore, it has been proposed that ligand recognition by integrins leads to structural alterations in the integrin cytoplasmic domain facilitating postligand binding events important for cell spreading and motility. While extensive studies have identified sequences within the extracellular domain of integrins involved in ligand binding (Smith and Cheresh, 1988, 1990; D'Souza et al., 1988, 1990; 1994; Loftus et al., 1990; Takada et al., 1992; Lee et al., 1995), little is known about the structural regions within the integrin cytoplasmic domain that mediate postligand binding events.

Integrin αvβ3 is highly expressed on various motile cell types in vivo. For example, this integrin is preferentially expressed on invasive melanoma cells (Albelda et al., 1991; Nip et al., 1993), angiogenic vascular cells (Brooks et al., 1994*a*,*b*) and on migratory neural crest cells (Delannet et al., 1994). Also, integrin αvβ3 readily promotes migration of primary endothelial cells or various transformed cells in vitro (Leavesley et al., 1992, 1993). Therefore, we designed experiments to identify regions within the cytoplasmic domain of αvβ3 that were involved in the cell motility response. To accomplish this objective, we used CS-1 melanoma cells that fail to express any vitronectin binding integrin due to their lack of β3 or β5 protein. We show in this report that cells transfected with a cDNA encoding full-length β3 express αvβ3 and thereby gain the ability to attach, spread, and migrate on a vitronectin substrate. To identify functional domains within the β3 cytoplasmic tail critical for these vitronectin adhesion events, CS1 cells were transfected with truncated or mutant forms of β3. In particular, we focused on two structural motifs, NPXY (744–747) and NXXY (756–759), which are predicted to form β turns in the cytoplasmic tails of, not only β3, but most of the other integrin β subunits (Fig. 1).

This mutational analysis of the β3 subunit demonstrates that disruption or elimination of the NPXY sequence prevents αvβ3-dependent CS-1 cell attachment or migration on a vitronectin substrate. We provide evidence that these mutations do not influence the soluble ligand binding properties of this receptor since either vitronectin- or RGD-containing peptides were able to interact equally well with all mutant forms of αvβ3 tested. Therefore, we propose that mutations in the NPXY motif of β3 perturb postligand binding events involved in αvβ3-dependent cytoskeletal assembly required for cell spreading and migration on a vitronectin substrate. This contention is supported by the finding that cells expressing a mutant or deleted NPXY sequence attached, but failed to spread or migrate on immobilized anti-β3 antibody.

Prior reports have shown that mutations in the β subunit cytoplasmic tail impact cell adhesion to immobilized ligand. Mutations in the β2 subunit cytoplasmic tail abolish αLβ2-mediated cell adhesion to purified ICAM (Hibbs et al., 1991). More recently, it was shown that deletion of the NPXY sequence expressed in the context of the platelet receptor αIIbβ3 altered the affinity state of this receptor for its ligand (O'Toole et al., 1995). Our studies suggest that NPXY mutations in αvβ3 have little or no effect on the affinity of this receptor for soluble ligand as measured by LIBS epitope expression in the presence of varying concentration of soluble RGD. Rather, this mutation appears to abolish the interaction of cells with immobilized ligand. These results may be explained by the inability of these mutant receptors to organize the actin cytoskeleton and promote a shape change on immobilized vitronectin or anti-$\beta_3$ antibody.

The NPXY sequence is present in the cytoplasmic domain of a number of non-integrin receptors such as the low density lipoprotein receptor where it has been linked to receptor internalization since mutations in NPXY result in reduced receptor internalization (Chen et al., 1990). CS-1 cells expressing αvβ3.741(Δ) lacking the NPXY sequence were tested for their ability to internalize integrin αvβ3 in response to cross-linking with anti-β3 antibodies. Interestingly, both the wild-type and NPXY-deleted receptors showed equivalent internalization (Filardo, E. J., unpublished data). These data suggest that while the NPXY is critical for the internalization of some cell surface receptors this motif does not appear to be required for αvβ3-dependent internalization.

Integrin-mediated cell adhesion is characterized by integrin clustering at discrete membrane sites known as focal contacts, which serve to strengthen the interaction between cells and their substratum (Burridge et al., 1988). In fact, focal contacts serve to tether actin filaments and stress fibers facilitating cell spreading. Based on previous studies, it is apparent that specific regions of the integrin β subunit cytoplasmic domain are critical for focal contact assembly (Reszka et al., 1992; Cone et al., 1994). This process likely depends on the ability of the integrin cytoplasmic tail to associate with various cytoplasmic protein such as talin and α-actinin (Tapley et al., 1989; Otey et al., 1992). In addition, there are several nonreceptor tyrosine kinases that are associated with focal contacts, including focal adhesion kinase, pp125FAK (Hanks et al., 1992; Kornberg et al., 1992; Schaller et al., 1992), src kinase, pp60v-src (Rohrschneider et al., 1990), type IV c-abl kinase (Van Etten et al., 1994), as well as a variety of SH2- and SH3-containing proteins (Miyamoto et al., 1995). These observations suggest that the integrin cytoplasmic domain is critical in recruiting signaling molecules that not only regulate cell shape, but also impact other events such as cell migration, invasion, and proliferation. To this end, we observed that the same mutations in β3 that prevent cell motility in vitro directly impact the metastatic properties of these cells in vivo. We showed that CS-1 melanoma cells lacking αvβ3 form large primary tumors when allowed to grow on the chick embryo CAM, yet these cells fail to establish lung metastases in these animals. However, transfection of CS-1 cells with wild-type β3 enables them to invade and colonize the lungs of these animals. Interestingly, primary tumors comprised of αvβ3 expressing CS-1 cells are fivefold smaller than their nontransfected counterparts, suggesting that the metastasis observed was due to the ability of CS-1 cells expressing αvβ3 to migrate from the

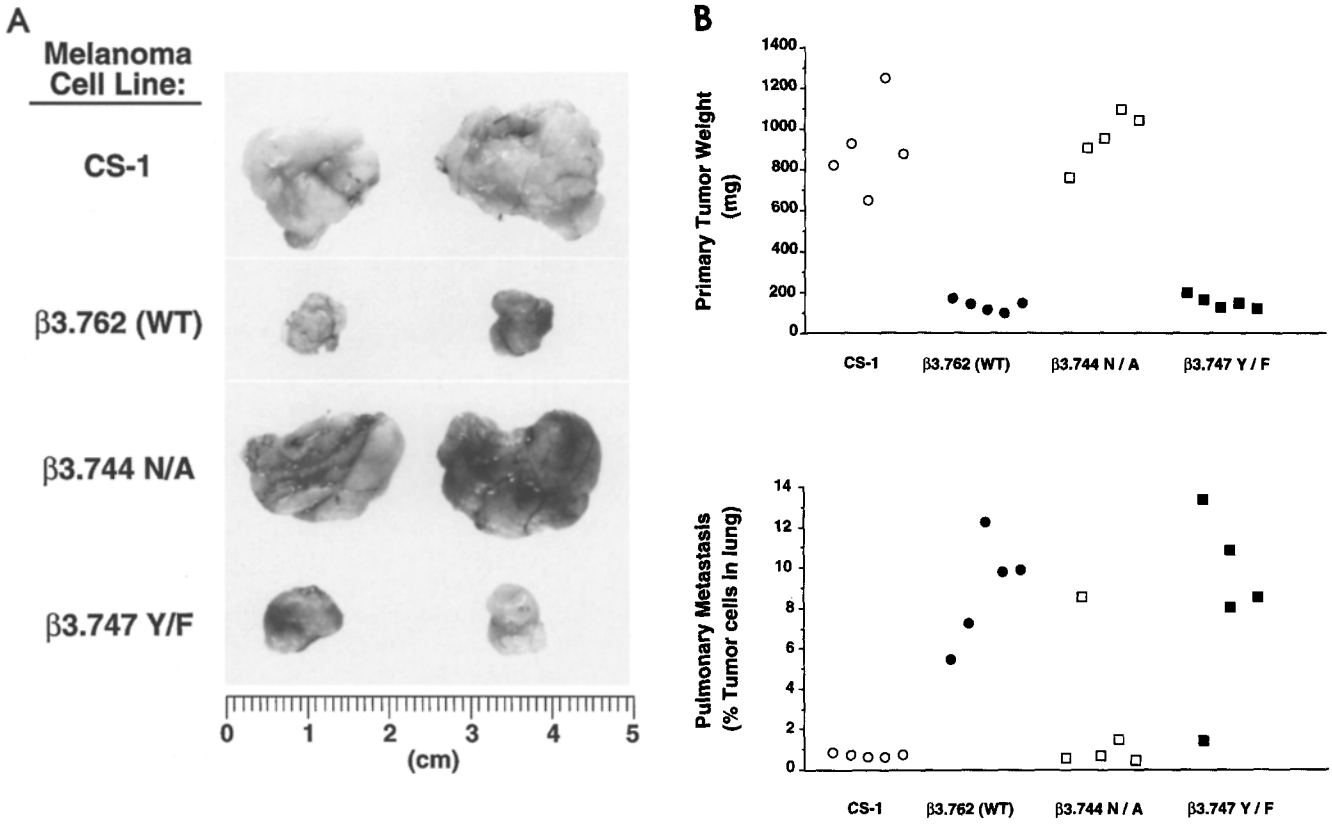


*Figure 6.* Effect of individual amino acid substitutions in the NPXY sequence within the β3 cytoplasmic tail on tumor growth and metastasis. (*A*) Representative examples of tumors excised from 17-d-old chick CAM after seven d of growth. (*B*) Primary tumor weight and percentage of metastatic melanoma cells present in chick lung after 7 d of tumor growth. Tumor weight is expressed in mg. The percentage of metastatic melanoma cells was determined by flow cytometry analysis of a single cell suspension prepared from whole lung using mAb, 7E2 specific for the hamster integrin β1 subunit. The difference in growth rate and metastasis are significant ($P \leq 0.001$) as determined by the Student *t* test.

site of the primary tumor. This contention is supported by the fact that these cell lines show identical growth rates in culture (data not shown) suggesting that the observed difference in the size of these primary tumors is not due to an intrinsic difference in the growth rate of these cells. More importantly, mutations in the NPXY sequence that prevent cell motility in vitro, show minimal metastasis in vivo, while forming large primary tumors identical to those formed by αvβ3-negative CS-1 cells. These findings are consistent with the observation that vertically invasive primary as well as metastatic human melanoma tissue expresses αvβ3, while noninvasive primary lesions lack αvβ3 (Albelda et al., 1991). Moreover, Nip and co-workers (1993) showed that melanoma cells isolated from nude mouse metastases express elevated levels of αvβ3 compared with their primary tumor cell counterparts.

The structural motif defined by the NPXY sequence in β3 is likely to play a major role in other integrin heterodimers since this motif is found in the same region of most other integrin β subunits. However, it is clear from our studies that the NPXY motif in the β3 subunit regulates postligand binding events that are critical to cellular morphology and migration in vitro. More importantly, we provide the first evidence of an integrin structural motif that plays a critical role in the metastasis of tumor cells in vivo. This is underscored by the finding that a single point mutation in the β3 subunit prevents αvβ3-dependent cellular post-ligand binding events leading to the metastatic behavior of tumor cells in vivo.

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